IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of :

Adrian Vivian Sinton HILL et al. :

Serial No. NEW : **Attn: Application Branch**

Filed January 11, 2002 : **Attorney Docket No. 2002_0026**

MALARIA PEPTIDES
(Rule 1.53(b) Continuation of Serial No. 08/714,175, Filed January 28, 1997)

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents,
Washington, DC 20231

Sir:

Kindly amend the application as follows:

IN THE SPECIFICATION

Page 1, immediately after the title, please insert:

This application is a continuation of Serial No. 08/714,175 filed January 28, 1997.

Page 1, line 6, please replace with the following:

BACKGROUND OF THE INVENTION

Page 1, line 24 to page 2, line 2, please rewrite the paragraph as follows:

This invention deals firstly with the identification of peptides from the malaria parasite *Plasmodium falciparum* which we identify as epitopes or potential epitopes for particular HLA class I molecules. A variety of evidence (cited in references 1 and 16 on page 16 hereinbelow) suggests that CTL play a role in immunity to this parasitic infection and disease by acting against

the liver-stage of infection. Thus these epitopes will be of value for inclusion in vaccines designed to provide immunity to *Plasmodium falciparum*. Furthermore, this work identifies for the first time CTL epitopes in the *P. falciparum* antigen, thrombospondin-related anonymous protein (TRAP; Robson et al., 1988), and thus identifies TRAP, and/or peptides from TRAP, as a useful component of a CTL-inducing vaccine against *P. falciparum* malaria.

Page 2, line 8, please replace with the following:

SUMMARY OF THE INVENTION

Page 3, line 10, please insert the following:

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT(S)

Page 3, line 13, please replace the paragraph as follows:

We have recently described a novel approach to identifying CTL epitopes and potential CTL epitopes in *P. falciparum* (Hill et al., 1992). This consisted, in brief, of 1) determining a motif for peptides bound to a particular HLA class I molecules, 2) synthesizing peptides from *P. falciparum* antigens congruent with this motif, 3) testing whether these peptides bind to that HLA class I allele using a binding assay known as a HLA assembly assay, and 4) testing whether lymphocytes from individuals exposed to malaria could recognise these peptides as epitopes after suitable in vitro restimulation and culture. In that work we identified peptide epitopes and potential peptide epitopes for two HLA class I molecules, HLA-B53 and HLA-B35. Here we extend that work to four further HLA class I molecules: HLA-A2, HLA-B8, HLA-B7 and HLA-B17. 52 peptides are identified that are shown either to be epitopes or potential epitopes for these HLA class I molecules.

Page 3, line 33 to page 4, line 26, please rewrite the paragraph as follows:

Motifs for eluted peptides.

The peptide binding motifs of HLA-A2, HLA-B7 and HLA-B8 have been described. For HLA-A2 strong preferences were found at positions 2 and 9 of bound peptides: for leucine, isoleucine and methionine at position 2, and for valine, leucine and isoleucine at position 9 (Falk et al., 1991 and Hunt et al., 1992). For HLA-B7 the preferred residues were proline at position 2 and a hydrophobic residue at position 9. Leucine, isoleucine, valine, alanine, phenylalanine and tryptophan were the hydrophobic residues preferred (Huczko et al., 1993). For HLA-B8 preferred residues were found at positions 3, 5 and 9 (Sutton et al., 1993). At positions 3 and 5 lysine or arginine are preferred and at position 9, leucine or isoleucine or valine. The motif for peptides bound to HLA-B17 has not been reported so we determined this exactly as described for HLA-B35 (Hill et al., 1992), using instead of the cell line CIR-B35, the cell line CIR-B58. Hence the peptide motif determined is strictly for HLA-B58 which along with HLA-B57 constitutes a subtype of HLA-B17. The primary amino acid sequences of HLA-B57 and HLA-B58 are very similar indicating that the types of peptides bound are likely to be very similar, at least in their key anchor residues. Hence we refer to the motif determined for CIR-B58 as a motif for HLA-B17. For HLA-B17, preferred amino acids were observed at positions 2 and 9: serine and threonine were preferred at positions 2 and a hydrophobic residue (defined as for B7 above) at position 9.

Page 4, line 29 to page 5, line 5, please rewrite the paragraph as follows:

As described previously for identification of HLA-B35 and HLA-B53 epitopes (Hill et al., 1992), peptides were synthesised to correspond to these four motifs from the primary amino acid sequences of four *P. falciparum* pre-erythrocytic antigens: CSP, TRAP, LSA-1 and SHEBA. Over one hundred peptides were synthesized. Binding assays (Elvin et al., 1992) were performed on selected peptides to determine whether they bound to HLA-A2, -B7, -B8, or -B58 using the untransfected T2 cell line (for HLA-A2) or the T2 cell line transfected with HLA-B7 (used to assess binding to HLA-B7s), or with HLA-B8 (used to assess binding to HLA-B8), or with HLA-B58 (used to assess binding to HLA-B58).

Page 5, line 8, please rewrite the paragraph as follows:

Peptides shown to bind to a particular HLA class I allele were tested for CTL recognition in assays using lymphocytes from malaria-exposed Gambians, as described previously (Hill et al., 1992). A minority of peptides, synthesized to correspond to a peptides binding motif were not tested in the relevant assembly assay but tested only for CTL recognition. Cells from 82 adult Gambians and 53 Gambian children, all exposed to malaria, were used in the course of these studies. The children and adults were HLA typed using cellular or molecular techniques as described previously (Hill et al., 1992). Peptides were incubated with cells either singly, or in pools as described (Hill et al., 1992) at concentrations of 10-100μM. Peptides could either be left with the cells for the duration of restimulation (as reported in Hill et al., 1992) or washed off after an hour. Cells were cultured for 1-3 weeks before a standard CTL chromium release assay (Hill et al., 1992) was performed using HLA matched or autologous B cell line targets pre-pulsed with the peptide to be tested. Peptides were tested either singly or in pools for CTL recognition. 10% or greater was regarded as a significant level of specific lysis.

Page 6, line 20, please rewrite the paragraph as follows:

ii) HLA-B7. CTL from one child with HLA-B7 showed significant lysis of HLA-B7 matched target cells pre-pulsed with the peptide pool cp6, cp6.1 and cp6.2. These peptides are encoded by allelic variants of the same region of the circumsporozoite protein gene (Doolan et al., 1992) identifying this sequence as an HLA-B7 restricted epitope. All three peptides bound to HLA-B7 in the HLA assembly assay. CTL from one adult (Z174) also recognised a pool of four peptides containing cp6, cp6.1, cp6.2 and cp21.

Page 8, line 29 to page 9, line 2, please rewrite the paragraph as follows:

This work identifies TRAP as a *P. falciparum* antigen which induces cytotoxic T lymphocyte responses in individuals exposed to endemic malaria. TRAP is expressed on sporozoites (Cowan et al.) as well as blood-stage malaria parasite and will therefore be present in the infected liver cell. We show here that TRAP contains CTL epitopes for three very common

HLA class I antigens (HLA-A2, -B8 and -B17) and therefore the induction of CTL to TRAP may be an important requirement of an effective CTL-inducing vaccine against *P. falciparum*.

Page 9, line 3, please rewrite the paragraph as follows:

As there is evidence that better CTL responses may be induced *in vivo* using epitopes or epitopes with a limited amount of flanking sequence than by using the whole antigen, the epitopes used here may be particularly valuable for the induction of CTL responses *in vivo* (Lawson et al., 1994).

Page 9, line 9, please rewrite the paragraph as follows:

This specification identifies for the first time the existence of CTL responses to the antigen TRAP in humans exposed to *P. falciparum* parasites. It is known from studies of rodent malaria and indirectly from studies of human *P. falciparum* malaria (Calvani et al., 1994) that CTL are likely to play a protective role but the target antigens of these CTL have been unclear. By identifying TRAP as a target of CTL responses in humans we identify it as a favourable antigen for inclusion in a vaccine designed to induce protective CTL responses. Moreover, we show here that TRAP contains conserved CTL epitopes for the very common class I antigen, HLA-A2, which is the most prevalent HLA-A or -B molecule in Caucasians, making TRAP of particular importance for immunization through CTL of Caucasian populations.

Page 9, line 24 to page 10, line 12, please rewrite the paragraph as follows:

There are several means by which the CTL epitopes identified here may be used to stimulate an immune response *in vivo* in humans. Either the peptides, or longer peptides containing them, can be used alone or with an adjuvant, such as incomplete Freund's adjuvant (Kast et al., 1991) or QS-21 (Newman et al., 1992) or NAGO (Zheng et al., 1992) or AF (Raychaudhuri et al., 1992), or as peptides with a lipid-tail added (Deres et al., 1989), a means that has been shown to enhance CTL induction *in vivo*. Alternatively, the epitopes can be delivered by recombining nucleotides encoding them into a gene coding for a particle such as a

recombinant Ty-virus-like-particle (Layton et al., 1993) or a recombinant hepatitis B virus antigen particle (Tindle et al., 1994). Alternatively, nucleotides encoding these epitopes can be incorporated into a recombinant virus such as a vaccinia virus or an attenuated vaccinia virus (Cox et al., 1993). Another means is to generate a recombinant bacterium such as a recombinant *Salmonella* containing nucleotides encoding these epitopes (Chatfield et al., 1992). Another means is to incorporate nucleotides encoding the epitopes identified into an expression vector, such as a DNA vaccine (Ulmer et al., 1993), that can express these epitopes after immunization. Finally, ribonucleotides coding for these epitopes can be used as an RNA-based vaccine (Martinon et al., 1993) to express these epitopes *in vivo*.

Page 10, line 16, please rewrite the paragraph as follows:

Although secondary (or recall) CTL responses to a variety of infectious micro-organisms can now be detected (e.g. as described for malaria above), CTL cannot be grown in this way from individuals unexposed to antigen or microorganism (Hill et al., 1992 and unpublished data). We describe here a novel method of growing "primary" CTL i.e. from previously unprimed individuals. This method can be employed for generating cell lines and clones which may be useful in various ways: to identify potential epitopes amongst a pool of peptides which bind to an HLA class I molecule; to identify peptides presented by HLA molecules on the surface of a cell using a CTL assay; for *in vivo* therapeutic use for the treatment of infectious or neoplastic disease. We demonstrate this method by describing the generation of CTL lines and clones to two peptides from *P. falciparum* from the lymphocytes of three individuals who have not been exposed to or infected by this parasite.

Page 10, line 35 to page 11, line 9, please rewrite the paragraph as follows:

The method described here for inducing primary CTL responses *in vitro* may be particularly useful in cancer immunotherapy. Studies in mice have demonstrated the potential of therapy with *ex vivo* cultured CTL (Greenberg et al., 1991), and human tumor-specific CTL have been identified in the peripheral blood or tumor-infiltrating lymphocytes from patients with

melanoma and renal cell carcinoma (Cerottini et al., 1992 and Koo et al., 1993). Additionally, induction of CTL against viral antigen epitopes *in vitro* may be useful in the therapy of viral infections such as HIV (Riddell et al., 1994).

Page 11, line 12, please rewrite the paragraph as follows:

Peripheral blood mononuclear cells from individuals never exposed to malaria were separated on Ficoll-hypaque and prepulsed for 2 hours with 20-100μM peptide. In the case of two HLA-A2 individuals the peptide was cp36; in the case of one HLA-B8 individual the peptide was tr43. The cells were then washed once and 5 million cells were incubated in a 2 ml well (in a standard humidified incubator with 5% CO_2) in α-MEM (minimal essential medium, GIBCO, UK) with autologous heat-inactivated human serum and 2μg per ml of keyhole limpet haemocyanin (KLH, Calbiochem, California, USA). The addition of the latter was based on our previous showing that this preferentially stimulates the $CD45RA^+$ (native) subset of CD4 T lymphocytes (Plebanski et al., 1992 and Plebanski et al., 1994). This CD4 T lymphocyte subset has been shown previously to promote CD8 T cell activity (Morimoto et al., 1986).

Page 13, please replace in its entirety with the following new page 13:

TABLE

label	SEQ ID No.	Sequence										Position
HLA-A2		1	2	3	4	5	6	7	8	9	10	
tr26 *	1	H	L	G	N	V	K	Y	L	V		3
tr29	2	L	L	M	D	C	S	G	S	I		51
tr39 *	3	G	I	A	G	G	L	A	L	L		500
ls10	4	I	L	Y	I	S	F	Y	F	I		4
ls11	5	Y	I	S	F	Y	F	I	L	V		6
ls19	6	G	I	Y	K	E	L	E	D	L		1801
ls23	7	H	I	F	D	G	D	N	E	I		1883
cp36 *	8	Y	L	K	T	I	Q	N	S	L		334
cp37 *	9	Y	L	Q	K	I	Q	N	S	L		334
cp38 *	10	Y	L	Q	K	I	K	N	S	L		334
cp39 *	11	Y	L	N	K	I	Q	N	S	L		334
HLA-B8												
cp43	12	L	R	K	P	K	H	K	K	L		134
cp44	13	L	K	K	I	K	N	S	I	S		335
cp45	14	Q	V	R	I	K	P	G	S	A		358
cp46	15	A	N	K	P	K	D	G	L	D		366
tr42 *	16	A	S	K	N	K	E	K	A	L		107
tr43 *	17	K	N	K	E	K	A	L	I	I		109
HLA-B7												
cp6 *	18	M	P	N	D	P	N	R	N	V		300
cp6.1 *	19	M	P	N	Y	P	N	R	N	V		300
cp6.2 *	20	M	P	N	N	P	N	R	N	V		300
ls6	21	K	P	I	V	Q	Y	D	N	F		1786
sh1	22	I	P	S	L	A	L	M	L	I		7
sh6	23	M	P	L	E	T	Q	L	A	I		77
cp21	24	N	P	D	P	N	A	N	P	N	V	120
tr6	25	N	P	E	N	P	P	N	P	D	I	348
tr13	26	I	P	D	S	I	Q	D	S	L		164
tr15	27	E	P	A	P	F	D	E	T	L		529
tr21	28	G	P	F	M	K	A	V	C	V		228

Page 14, please replace in its entirety the following new page 14:

label	SEQ ID No.	Sequence										Position
		1	2	3	4	5	6	7	8	9	10	
HLA-B17												
cp48	29	L	S	V	S	S	F	L	F	V		8
cp55	30	G	S	A	N	K	P	K	D	E	L	364
cp56	31	C	S	S	V	F	N	V	V			388
ls36	32	N	S	E	K	D	E	I	I			28
ls37	33	G	S	S	N	S	R	N	R	I		42
ls39	34	V	S	Q	T	N	F	K	S	L		92
ls40	35	K	S	L	L	R	N	L	G	V		98
ls42	36	Q	S	D	S	E	Q	E	R	L		179
ls45	37	R	T	K	A	S	K	E	T	L		1187
ls48	38	H	T	L	E	T	V	N	I			1742
ls49	39	I	S	D	V	N	D	F	Q	I		1749
ls50	40	I	S	K	Y	E	D	E	I			1757
ls51	41	I	S	A	E	Y	D	D	S	L		1764
ls53	42	K	S	L	Y	D	E	H	I			1854
ls54	43	L	S	E	D	I	T	K	Y	F		1898
ls55	44	T	K	Y	F	M	K	L				1902
tr57	45	K	T	A	S	C	G	V	W	D	EW	240
tr58	46	G	T	R	S	R	K	R	E	I	L	260
tr59	47	S	S	V	Q	K	P	E	E	N	I	311
tr60	48	D	S	E	K	E	V	P	S	D	V	367
tr61	49	Y	S	P	L	P	P	K	V	L		415
tr62	50	E	S	D	N	K	Y	K	I	A		490
tr63	51	A	T	P	Y	A	G	E	P	A		523
tr64	52	E	T	L	G	E	E	D	K	D	L	535

* Peptide identified as an epitope for a secondary cytotoxic T lymphocyte response.

Page 15, please rewrite the paragraph as follows:

Table: Peptides from four *Plasmodium falciparum* antigens, circumsporozoite protein (cp), thrombospondin-related anonymous protein (tr) spirozoite hepatocyte binding antigen (sh) and liver-stage antigen-1 (ls), that are here identified as CTL epitopes or as potential CTL epitopes for particular HLA class I molecules. Epitopes are shown in bold type. The position of the first amino acid of the peptide in the published amino acid sequence (CSP - Dame et al., 1984; LSA-1 - Zhu et al., 1991; TRAP - Robson et al., 1988) is shown. Note that tr57 is 11 amino acids in length. The standard one letter amino acid code is used.

IN THE CLAIMS

Cancel without prejudice claims 1-11.

Kindly add the following new claims:

12. **(New)** An isolated peptide consisting of between 9 and 100 contiguous amino acids of the thrombospondin related anonymous protein (TRAP) malarial antigen, including the amino acid sequence of at least one of SEQ ID Nos. 1 or 17, said amino acid sequence of SEQ ID No. 1 being capable of binding to human leukocyte antigen HLA-A2 when said sequence is present in said isolated peptide, said amino acid sequence of SEQ ID No. 17 being capable of binding to human leukocyte antigen HLA-B8 when said sequence is present in said isolated peptide, and said sequences being recognized by cytotoxic T lymphocytes (CTLs) from individuals currently or previously infected by Plasmodium when one or both sequences are present in said isolated peptide.

13. **(New)** The isolated peptide according to claim 12, comprising both amino acid sequences of SEQ ID Nos. 1 and 17.

14. **(New)** The isolated peptide according to claim 12, wherein the peptide has an N-terminus or C-terminus having a covalently bound immunogenicity enhancing lipid tail.

15.**(New)** The isolated peptide according to claim 13, wherein the peptide has an N-terminus or C-terminus having a covalently bound immunogenicity enhancing lipid tail.

16. **(New)** A vaccine for immunization against malaria, said vaccine comprising an effective amount of at least one peptide according to claim 12 together with a pharmaceutically acceptable carrier.

17. **(New)** A vaccine for immunization against malaria, said vaccine comprising an effective amount of at least one peptide according to claim 13 together with a pharmaceutically acceptable carrier.

18. **(New)** A method for immunizing against malaria, which method comprises administering to a patient in need thereof an effective amount of an isolated peptide according to claim 12, together with a pharmaceutically acceptable carrier.

IN THE SEQUENCE LISTING

Please replace the paper copy of the Sequence Listing with the attached substitute Sequence Listing.

REMARKS

The present paper is submitted concurrently with a Rule 53(b) continuation application of Serial No. 08/714,175.

The present paper amends the specification along the lines of the amendments to the specification of the parent application.

The amendment cancels without prejudice original claims 1-11 and adds new claims 12-18. Claims 12-18 correspond to claims 12-18 in the parent application, except claim 12 has been further amended in response to the Advisory Action dated December 5, 2001. Specifically, claim 12 is amended to clarify that the TRAP antigen is a thrombospondin related anonymous protein (TRAP) malarial antigen. This is supported in the specification for example at page 1, lines 35-36 and page 2, line 2. Claim 12 is further amended to ensure that the meanings of the acronyms "HLA" and "CTLS" are clear.

In view of these amendments, it is clear what antigens are encompassed by this term.

It is furthermore considered to be clear that the TRAP is a single protein as defined by Robson et al. See the specification at page 1, lines 35-36 and page 15, line 10. Hence there is no new matter or written description issue raised.

Although non-elected, claim 18 is again presented and has been amended to correspond to pending claim 12. Accordingly, upon an allowance of claims 12-17, claim 18 should be rejoined under PTO rules.

Finally there is submitted therewith a substitute Sequence Listing in paper and computer readable form. The paper and computer readable copies are identical. No new matter is added.

Favorable action and allowance is solicited.

Respectfully submitted,

Adrian Vivian Sinton HILL et al.

By ______________________

Warren M. Cheek, Jr.
Registration No. 33,367
Attorney for Applicants

WMC/dlk
Washington, D.C. 20006-1021
Telephone (202) 721-8200
Facsimile (202) 721-8250
January 11, 2002

MALARIA PEPTIDES

This application is a continuation of Serial No. 08/714,115 filed January 28, 199

BACKGROUND OF THE INVENTION

INTRODUCTION

The identification of peptide epitopes for HLA class I molecules is of importance for several areas of biomedical science. Firstly, such epitopes are the central components of vaccines designed to provide immunity mediated by cytotoxic T lymphocytes (CTL) to infectious microorganisms. This is a consequence of the method whereby cytotoxic T lymphocytes function: they recognise a peptide epitope of 8-11 amino acids in length presented by an HLA class I molecule. Secondly, there is increasing interest in the possibility that neoplastic tumours might be ameliorated or cured by inducing, either in vivo or in vitro, CTL that can recognize tumour-specific peptides on HLA class I molecules. Thirdly, the identification of such epitopes recognised in autoimmune disorders would provide insights into pathogenesis and suggest new specific methods of treating these disorders.

This invention deals firstly with the identification of peptides from the malaria parasite *Plasmodium falciparum* which we identify as epitopes or potential epitopes for particular HLA class I molecules. A variety of evidence (cited in references 1 and 16 on page 16 hereinbelow) suggests that CTL play a role in immunity to this parasitic infection and disease by acting against the liver-stage of infection. Thus these epitopes will be of value for inclusion in vaccines designed to provide immunity to *Plasmodium falciparum*. Furthermore, this work identifies for the first time CTL epitopes in the *P. falciparum* antigen, thrombospondin-related anonymous protein ~~(TRAP)~~ (TRAP; Robson et al., 1988), and thus identifies TRAP, and / or

peptides from TRAP, as a useful component of a CTL-inducing vaccine against *P. falciparum* malaria.

This invention deals secondly with a novel method of identifying cytotoxic T lymphocyte epitopes and of producing CTL lines and clones by the use of a new method of inducing CTL responses *in vitro*.

SUMMARY OF THE INVENTION

In one aspect the invention provides the 8 to 11-mer peptides set out in the table below, being either epitopes or potential epitopes for the stated HLA class I molecules, conservative variants thereof, and longer peptides containing these sequences which are sub-units of the indicated antigens. It is envisaged that these peptides are generally 8 to 100 amino acid residues in length, and that the sequence shown in the table is the main functional epitope present. No per se claim is made to the antigen as a whole, nor to any fragment which constitutes the larger part of the antigen.

Two or more of these peptides may be joined together in sequence. The peptide may have an N- or C-terminus carrying a lipid tail, a modification known to enhance CTL induction *in vivo*.

Also included are oligonucleotides which code for the stated peptides. The term oligonucleotide is here used to encompass nucleic acid chains of about 24 to about 300 nucleotide residues.

Also included are vaccines comprising peptides or oligonucleotides as defined, for immunisation against malaria.

In another aspect the invention provides a method of inducing primary cytotoxic T lymphocyte responses to a chosen antigen or microorganism, which method comprises incubating lymphocytes *ex vivo* with the chosen antigen or microorganism in the presence of

KLH (keyhole limpet haemocyanin) or any other substance which preferentially stimulates a CD45RA$^+$ subset of T lymphocyte.

In this method the KLH or other adjuvant is used at a concentration suitable to stimulate the desired CTL response, which optimum concentration may readily be determined by experiment. Preferably IL-7 (interleukin-7) and/or IL-2 (interleukin-2) is also present during the said incubation.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT(S)

SECTION A

Identification of P. falciparum peptides and epitopes

We have recently described a novel approach to identifying CTL epitopes and potential CTL epitopes in *P. falciparum*[1]. (Hill et al., 1992) This consisted, in brief, of 1) determining a motif for peptides bound to a particular HLA class I molecules, 2) synthesizing peptides from *P. falciparum* antigens congruent with this motif, 3) testing whether these peptides bind to that HLA class I allele using a binding assay known as a HLA assembly assay, and 4) testing whether lymphocytes from individuals exposed to malaria could recognise these peptides as epitopes after suitable in vitro restimulation and culture. In that work we identified peptide epitopes and potential peptide epitopes for two HLA class I molecules, HLA-B53 and HLA-B35. Here we extend that work to four further HLA class I molecules: HLA-A2, HLA-B8, HLA-B7 and HLA-B17. 52 peptides are identified that are shown either to be epitopes or potential epitopes for these HLA class I molecules.

~~Detailed description~~

Motifs for eluted peptides.

The peptide binding motifs of HLA-A2, HLA-B7 and HLA-B8 have been described. For HLA-A2 strong

preferences were found at positions 2 and 9 of bound peptides: for leucine, isoleucine and methionine at position 2, and for valine, leucine and isoleucine at position 9[3,4] (Falk et al., 1991 and Hunt et al., 1992). For HLA-B7 the preferred residues were proline at position 2 and a hydrophobic residue at position 9. Leucine, isoleucine, valine, alanine, phenylalanine and tryptophan were the hydrophobic residues preferred[5] (Hunczko et al., 1993). For HLA-B8 preferred residues were found at positions 3, 5 and 9[6] (Sutton et al., 1993). At positions 3 and 5 lysine or arginine are preferred and at position 9, leucine or isoleucine or valine. The motif for peptides bound to HLA-B17 has not been reported so we determined this exactly as described for HLA-B35[1] (Hill et al., 1992) using instead of the cell line CIR-B35, the cell line CIR-B58. Hence the peptide motif determined is strictly for HLA-B58 which along with HLA-B57 constitutes a subtype of HLA-B17. The primary amino acid sequences of HLA-B57 and HLA-B58 are very similar indicating that the types of peptides bound are likely to be very similar, at least in their key anchor residues. Hence we refer to the motif determined for CIR-B58 as a motif for HLA-B17. For HLA-B17, preferred amino acids were observed at positions 2 and 9: serine and threonine were preferred at position 2 and a hydrophobic residue (defined as for B7 above) at position 9.

Peptide Synthesis and Binding Assays

As described previously for identification of HLA-B35 and HLA-B53 epitopes[1] (Hill et al., 1992), peptides were synthesised to correspond to these four motifs from the primary amino acid sequences of four *P. falciparum* pre-erythrocytic antigens: CSP, TRAP, LSA-1 and SHEBA. Over one hundred peptides were synthesized. Binding assays[1] (Elvin et al., 1992) were performed on selected peptides to determine whether they bound to HLA-A2, -B7, -B8, or

-B58 using the untransfected T2 cell line (for HLA-A2) or the T2 cell line transfected with HLA-B7 (used to assess binding to HLA-B7s), or with HLA-B8 (used to assess binding to HLA-B8), or with HLA-B58 (used to assess binding to HLA-B58).

Cytotoxic T Lymphocyte assays

Peptides shown to bind to a particular HLA class I allele were tested for CTL recognition in assays using lymphocytes from malaria-exposed Gambians, as described previously[1]. (Hill et al., 1992) A minority of peptides, synthesized to correspond to a peptides binding motif were not tested in the relevant assembly assay but tested only for CTL recognition. Cells from 82 adult Gambians and 53 Gambian children, all exposed to malaria, were used in the course of these studies. The children and adults were HLA typed using cellular or molecular techniques as described previously[1]. (Hill et al., 1992) Peptides were incubated with cells either singly, or in pools as described[1] (Hill et al., 1992) at concentrations of 10-100μM. Peptides could either be left with the cells for the duration of restimulation (as reported[1] in Hill et al., 1992) or washed off after an hour. Cells were cultured for 1-3 weeks before a standard CTL chromium release assay[1] (Hill et al., 1992) was performed using HLA matched or autologous B cell line targets pre-pulsed with the peptide to be tested. Peptides were tested either singly or in pools for CTL recognition. 10% or greater was regarded as a significant level of specific lysis.

Peptides Identified: CTL Epitopes.

The sequences of all peptides referred to are shown in the table.

i) HLA-A2. Two Gambian individuals showed positive CTL responses to HLA-A2 peptides. One individual (Z62) recognised a pool of three TRAP

peptides, tr26, tr29 and tr39. Insufficient cells were available to determine which of these three peptides was the epitope. However a second adult (Z60) also recognised this pool of three peptides and subsequently showed significant lysis of tr39- but not tr26-, nor tr29-pulsed target cells. Hence tr39 is an HLA-A2-restricted epitope. Both individuals were of the HLA-A*0201 subtype of HLA-A2. All three of these peptides bound to HLA-A2 in the assembly assay.

Further studies consisting of cytotoxic T lymphocyte assays using lymphocytes from malaria-exposed Gambians have confirmed that the following peptides listed in the table are cytotoxic T lymphocyte epitopes. For individuals with HLA-A2, CTL from at least one individual have been found to recognise the peptides tr26, tr29, cp36, cp37, cp38 and cp39. These epitopes for HLA-A2 are in addition to tr39 identified previously.

ii) HLA-B7. CTL from one child with HLA-B7 showed significant lysis of HLA-B7 matched target cells pre-pulsed with the peptide pool cp6, cp6.1 and cp6.2. These peptides are encoded by allelic variants of the same region of the circumsporozoite protein gene[8] (Doolan et al., 1992) identifying this sequence as an HLA-B7 restricted epitope. All three peptides bound to HLA-B7 in the HLA assembly assay. CTL from one adult (Z174) also recognised a pool of four peptides containing cp6, cp6.1, cp6.2 and cp21.

iii) HLA-B8. CTL from one adult (Z42) showed significant lysis of a pool of peptides containing tr42, tr43, tr44 and tr45. Two of these four peptides, tr42 and tr43 bound to HLA-B8 in the HLA assembly assay. Subsequently the same CTL line showed specific lysis of tr42 only defining this as an HLA-B8-

I molecule indicates that they are likely to be presented on the surface of malaria-infected hepatocytes *in vivo*. Hence, even if detectable levels of CTL to these peptides are not found in malaria-exposed individuals, induction of CTL to these peptides may be a useful means of immunizing against *P. falciparum* malaria.

i) HLA-A2. The following peptides were shown to bind to HLA-A2 in the HLA assembly assay and are therefore potential CTL epitopes: ls10, ls11, ls19, ls23. The peptide cp36 was also capable of inducing a primary CTL response in malaria-unexposed individuals (see below).

ii) HLA-B7. The following peptides were found to bind to HLA-B7 in the HLA assembly assay and are therefore potential epitopes: cp21, ls6, tr6, tr13, tr15, tr21, sh1, sh6.

iii) HLA-B17. The following peptides were found to bind to HLA-B17 in the HLA assembly assay and are therefore potential epitopes: cp48, cp55, cp56, and all the 13 ls peptides listed above as being present in the pool recognised by two individuals with HLA-B17.

Further evidence for the potential usefulness of the peptides tr43, cp36 and variants of cp36 (cp37-39) in a malaria vaccine is presented below where we show that CTL may be generated *in vitro* against these peptides from malaria un-exposed individuals.

This work identifies TRAP as a *P. falciparum* antigen which induces cytotoxic T lymphocyte responses in individuals exposed to endemic malaria. TRAP is expressed on sporozoites (Cowan et al.) as well as blood-stage malaria parasite and will therefore be present in the infected liver cell. We show here that TRAP contains CTL epitopes for three very common HLA class I antigens (HLA-A2, -B8 and -B17) and therefore the induction of

CTL to TRAP may be an important requirement of an effective CTL-inducing vaccine against *P. falciparum*.

As there is evidence that better CTL responses may be induced *in vivo* using epitopes or epitopes with a limited amount of flanking sequence than by using the whole antigen, the epitopes used here may be particularly valuable for the induction of CTL responses *in vivo*[15]. (Lawson et al., 1994)

This specification identifies for the first time the existence of CTL responses to the antigen TRAP in humans exposed to *P. falciparum* parasites. It is known from studies of rodent malaria and indirectly from studies of human *P. falciparum* malaria[16] (Calvani et al., 1994) that CTL are likely to play a protective role but the target antigens of these CTL have been unclear. By identifying TRAP as a target of CTL responses in humans we identify it as a favourable antigen for inclusion in a vaccine designed to induce protective CTL responses. Moreover, we show here that TRAP contains conserved CTL epitopes for the very common class I antigen, HLA-A2, which is the most prevalent HLA-A or -B molecule in Caucasians, making TRAP of particular importance for immunization through CTL of Caucasian populations.

There are several means by which the CTL epitopes identified here may be used to stimulate an immune response *in vivo* in humans. Either the peptides, or longer peptides containing them, can be used alone or with an adjuvant, such as incomplete Freund's adjuvant[17] or QS-21[18] or NAGO[19] or AF[20], or as peptides with a lipid-tail added[21], a means that has been shown to enhance CTL induction *in vivo*. Alternatively, the epitopes can be delivered by recombining nucleotides encoding them into a gene coding for a particle such as a recombinant Ty-virus-like-particle[22] or a recombinant hepatitis B virus antigen particle[23]. Alternatively, nucleotides

(Deres et al 1989)

(Rayshavshvili et al 1992)

(Zheng et al., 1992)

(Newman et al., 1992)

(Kast et al., 1991)

(Tindle et al., 1994)

(Ton et al., 1993)

encoding these epitopes can be incorporated into a recombinant virus such as a vaccinia virus or an attenuated vaccinia virus[24]. Another means is to generate a recombinant bacterium such as a recombinant *Salmonella* containing nucleotides encoding these epitopes[25]. Another means is to incorporate nucleotides encoding the epitopes identified into an expression vector, such as a DNA vaccine[26], that can express these epitopes after immunization. Finally, ribonucleotides coding for these epitopes can be used as an RNA-based vaccine[27] to express these epitopes *in vivo*.

(Cox et al., 1993)
(Chatfield et al., 1992)
(Ulmer et al., 1993)
(Martinon et al., 1993)

SECTION B

A Method of Inducing Primary CTL Responses In Vitro

Although secondary (or recall) CTL responses to a variety of infectious micro-organisms can now be detected (e.g. as described for malaria above), CTL cannot be grown in this way from individuals unexposed to antigen or microorganism (~~ref. 1~~ Hill et al., 1992 and unpublished data). We describe here a novel method of growing "primary" CTL i.e. from previously unprimed individuals. This method can be employed for generating cell lines and clones which may be useful in various ways: to identify potential epitopes amongst a pool of peptides which bind to an HLA class I molecule; to identify peptides presented by HLA molecules on the surface of a cell using a CTL assay; for *in vivo* therapeutic use for the treatment of infectious or neoplastic disease. We demonstrate this method by describing the generation of CTL lines and clones to two peptides from *P. falciparum* from the lymphocytes of three individuals who have not been exposed to or infected by this parasite.

The method described here for inducing primary CTL responses *in vitro* may be particularly

useful in cancer immunotherapy. Studies in mice have demonstrated the potential of therapy with *ex vivo* cultured CTL[28], and human tumor-specific CTL have been identified in the peripheral blood or tumor-infiltrating lymphocytes from patients with melanoma and renal cell carcinoma[29,30]. Additionally, induction of CTL against viral antigen epitopes *in vitro* may be useful in the therapy of viral infections such as HIV[31].

Detailed description

Peripheral blood mononuclear cells from individuals never exposed to malaria were separated on Ficoll-hypaque and prepulsed for 2 hours with 20-100μM peptide. In the case of two HLA-A2 individuals the peptide was cp36; in the case of one HLA-B8 individual the peptide was tr43. The cells were then washed once and 5 million cells were incubated in a 2 ml well (in a standard humidified incubator with 5% CO_2) in α-MEM (minimal essential medium, GIBCO, UK) with autologous heat-inactivated human serum and 2μg per ml of keyhole limpet haemocyanin (KLH, Calbiochem, California, USA). The addition of the latter was based on our previous showing that this preferentially stimulates the $CD45RA^+$ (naive) subset of CD4 T lymphocytes[10,11]. This CD4 T lymphocyte subset has been shown previously to promote CD8 T cell activity[12].

After 72 hours interleukin-2 (Cetus, California, USA) was added at a concentration of 10 units per ml and the cells were cultured for a further 4 days. Then 5000 of the cells were restimulated in 150μl of α-MEM with 10% autologous human serum with 100,000 irradiated autologous peripheral blood lymphocytes, that had been pre-pulsed for one hour in 20μM of the same peptide used for the first stimulation and then washed once. On the following day 3 units of

Version with Markings to Show Changes Made

TABLE

label		Seq ID No.	Sequence									Position
HLA-A2			1	2	3	4	5	6	7	8	9	10	
tr26	*	1	H	L	G	N	V	K	Y	L	V		3
tr29		2	L	L	M	D	C	S	G	S	I		51
tr39	*	3	G	I	A	G	G	L	A	L	L		500
ls10		4	I	L	Y	I	S	F	Y	F	I		4
ls11		5	Y	I	S	F	Y	F	I	L	V		6
ls19		6	G	I	Y	K	E	L	E	D	L		1801
ls23		7	H	I	F	D	G	D	N	E	I		1883
cp36	*	8	Y	L	K	T	I	Q	N	S	L		334
cp37	*	9	Y	L	Q	K	I	Q	N	S	L		334
cp38	*	10	Y	L	Q	K	I	K	N	S	L		334
cp39	*	11	Y	L	N	K	I	Q	N	S	L		334
HLA-B8													
cp43		12	L	R	K	P	K	H	K	K	L		134
cp44		13	L	K	K	I	K	N	S	I	S		335
cp45		14	Q	V	R	I	K	P	G	S	A		358
cp46		15	A	N	K	P	K	D	G	L	D		366
tr42	*	16	A	S	K	N	K	E	K	A	L		107
tr43	*	17	K	N	K	E	K	A	L	I	I		109
HLA-B7													
cp6	*	18	M	P	N	D	P	N	R	N	V		300
cp6.1	*	19	M	P	N	Y	P	N	R	N	V		300
cp6.2	*	20	M	P	N	N	P	N	R	N	V		300
ls6		21	K	P	I	V	Q	Y	D	N	F		1786
sh1		22	I	P	S	L	A	L	M	L	I		7
sh6		23	M	P	L	E	T	Q	L	A	I		77
cp21		24	N	P	D	P	N	A	N	P	N	V	120
tr6		25	N	P	E	N	P	P	N	P	D	I	348
tr13		26	I	P	D	S	I	Q	D	S	L		164
tr15		27	E	P	A	P	F	D	E	T	L		529
tr21		28	G	P	F	M	K	A	V	C	V		228

Version with Markings to Show Changes Made

label	SEQ ID NO.	Sequence									Position
		1	2	3	4	5	6	7	8	9	10	
HLA-B17												
cp48	29	L	S	V	S	S	F	L	F	V		8
cp55	30	G	S	A	N	K	P	K	D	E	L	364
cp56	31	C	S	S	V	F	N	V	V			388
ls36	32	N	S	E	K	D	E	I	I			28
ls37	33	G	S	S	N	S	R	N	R	I		42
ls39	34	V	S	Q	T	N	F	K	S	L		92
ls40	35	K	S	L	L	R	N	L	G	V		98
ls42	36	Q	S	D	S	E	Q	E	R	L		179
ls45	37	R	T	K	A	S	K	E	T	L		1187
ls48	38	H	T	L	E	T	V	N	I			1742
ls49	39	I	S	D	V	N	D	F	Q	I		1749
ls50	40	I	S	K	Y	E	D	E	I			1757
ls51	41	I	S	A	E	Y	D	D	S	L		1764
ls53	42	K	S	L	Y	D	E	H	I			1854
ls54	43	L	S	E	D	I	T	K	Y	F		1898
ls55	44	T	K	Y	F	M	K	L				1902
tr57	45	K	T	A	S	C	G	V	W	D	EW	240
tr58	46	G	T	R	S	R	K	R	E	I	L	260
tr59	47	S	S	V	Q	K	P	E	E	N	I	311
tr60	48	D	S	E	K	E	V	P	S	D	V	367
tr61	49	Y	S	P	L	P	P	K	V	L		415
tr62	50	E	S	D	N	K	Y	K	I	A		490
tr63	51	A	T	P	Y	A	G	E	P	A		523
tr64	52	E	T	L	G	E	E	D	K	D	L	535

* Peptide identified as an epitope for a secondary cytotoxic T lymphocyte response.

Version with Markings to Show Changes Made

Table: Peptides from four *Plasmodium falciparum* antigens, circumsporozoite protein (cp), thrombospondin-related anonymous protein (tr) spirozoite hepatocyte binding antigen (sh) and liver-stage antigen-1 (ls), that are here identified as CTL epitopes or as potential CTL epitopes for particular HLA class I molecules. Epitopes are shown in bold type. The position of the first amino acid of the peptide in the published amino acid sequence (CSP - Dame et al., 1984 ~~ref. 13~~; LSA-1 - ~~ref. 14~~; TRAP - ~~ref. 2~~) is shown. Note that tr57 is 11 amino acids in length. The standard one letter amino acid code is used.

Zhu et al., 1991

Robson et al., 1988